PHILIP MORRIS U. S. A.

INTER-OFFICE CORRESPONDENCE

RICHMOND, VIRGINIA

To: Mr. J. L. Charles Date: October 29, 1981

From: Karen Rapp Sherwood

Subject: Project Charge Number 6906 (Biological Effects of Smoke)--Plans and Goals for 1982

1. INTRODUCTION

The objectives of this project remain essentially unchanged.

a) To utilize the *Salmonella*/microsome assay in a battery of short-term *in vitro* assays to evaluate the potential effects of cigarette smoke products.

b) To conduct tests on potential new cigarette products or additives upon request and to assist in the evaluation and interpretation of the results obtained.

c) To conduct research investigations designed to generate an understanding of the nature and control of cigarette smoke product activity in the *Salmonella*/microsome assay.

2. PLANS AND GOALS FOR 1982--*SALMONELLA*/MICROSOME MUTATION ASSAY

The majority of these studies will involve continuing collaborative interactions with various 6908 and 6910 project personnel which are directed toward refining our understanding of the determinants of CSC activity in conjunction with other similar studies by projects 6902 and 6904, and testing on request from 6900 project personnel. We anticipate a significant increase in testing for both of these areas, especially requests for testing CSCs and/or potential cigarette additives by 6900 project personnel.

A. Assay Standardization and Quality Assurance

In order to keep the *Salmonella*/microsome assay up and running, several studies are necessary in or by the first quarter, 1982. These include: 1) a complete transition from the Sigma to the DEC system in order to retain all of our current computer capabilities for handling and storing our data; 2) preparation of a new TA98 frozen ampoule stock culture for use as inoculum in routine testing; and, 3) purchase and complete evaluation of a new batch of Aroclor®-1254 induced rat liver microsomes according to our SOP (for microsome evaluation) for use in routine testing. Since Aroclor®-induced hamster liver microsomes are, or soon will be available from project 6904, we would also like to evaluate them (at the same time that we evaluate the rat liver microsomes) for a preliminary data base

should we need to use them for testing/screening potential cigarette additives, fractions, *etc.*, in the future. By the end of the second or third quarter, 1982, the interface of the automatic colony counter directly to the main computer (with the assistance and services of CAD) will be completed in order to help us to handle our data more efficiently with our increased work load; and the "*Salmonella*/Microsome Assay Methods Manual" which documents our standardized operating procedures in this laboratory will be issued. As soon as the new *Salmonella typhimurium* tester strains which are reported to detect additional classes of biologically active compounds are available from Ames' laboratory, we are planning to obtain them for our bank of tester strains. Based on the literature on these strains and our own project priorities, we may evaluate them for their potential use in our laboratory. In addition, we will continue our efforts to carefully monitor the assay results on positive and negative control compounds, viable counts, and markers on a routine basis.

B. Additive and CSC Testing

Based on information provided by 6900 project personnel (R. A. Pages, R. D. Carpenter, and J. E. John), we are expecting an increase in requests for testing CSCs and/or potential cigarette additives in 1982 over those from 1981. As in the past, this area of work will continue to be conducted promptly and on a high priority basis.

C. Sidestream *versus* Mainstream Activity (with 6910)

Our major interest in determining the activity of sidestream *versus* mainstream CSC (or TPM), in this and other *in vitro* assays, is to try to relate any observed differences in activity to differences in CSC composition and/or to the different temperature/pyrosynthesis, *etc.*, conditions that are recognized for cigarette filler that is burned statically *versus* dynamically. The results of the work conducted to date show: 1) sidestream CSC or TPM is less active than mainstream CSC or TPM; 2) the activity of sidestream basic fraction is less than that of the mainstream basic fraction; 3) mainstream TPM is less active than mainstream IT CSC; 4) the activity of sidestream IT CSC from statically burned cigarettes is the same as that from cigarettes burned in the normal manner (58 sec. puff interval); and, 5) from preliminary 2R1 data, there is evidence that the sidestream TPM from statically burned cigarettes is more active than the sidestream IT CSC from statically burned, or dynamically smoked cigarettes. The experiments thus far have been restricted to the use of the 2R1 KRC, RCB, burley, bright, and RL reference cigarettes and some control RL base web and K^+ salt added samples on RL base web (KOAc and KCl). These studies will continue in 1982. Due to the high water content of sidestream samples, some further work will be done to better profile the dose range in order to try to increase our ability to measure sidestream activity in the assay, and to determine the best dose-response range for testing. Once the collection procedure for sidestream smoke, the TPM extraction procedure, and the method of preparing TPM stock solutions in DMSO is standardized, we plan to retest the five model cigarettes to compare sidestream *versus* mainstream TPM collected from the same cigarettes under static/dynamic burn

conditions. It is hoped that we will then be able to answer some of the questions on the absolute and relative sidestream *versus* mainstream activity. The results of these studies will further dictate what direction we will then take; *e. g.*, expand testing to other cigarette types; testing of additional sidestream fractions (acid/neutral, base, and 2AC/H/NH, *etc.*); and trying to relate activity to chemical composition.

D. Cigarette Filler Pyrolysis (with 6910 and 6908)

This area of investigation will continue to be expanded during 1982. On a continuing basis, we are seeking to define what relationships may exist between pyrolyzate and CSC activity and composition with a view toward ultimately being able to generate samples for testing when cigarette fabrication is difficult or impossible and/or to evaluate the major precursors of CSC activity. It may also be possible that the pyrolyzate work could lead to setting up an additional model system for studying sidestream activity. So far, the parameters involved in generating pyrolyzate samples that have been studied in 1981 are: large batch *versus* small batch; carrier gas flow rates; activity *versus* temperature from 300°-900°C in 100°C increments; air *versus* nitrogen; helium *versus* nitrogen; and 20 minute *versus* 12 minute pyrolysis time. Base fractions from 2R1 pyrolyzate were also tested. In 1982, we will continue to study the activity of pyrolyzate *versus* atmosphere and temperature for model cigarette filler types and dry *versus* conditioned filler; base fractions: pyrolyzate *versus* IT CSC activity (also, acid/neutral fractions eventually); and pyrolysis of salt effect filler samples. Testing of pyrolyzates from bright, burley, and 1:1 blended samples initiated in the fourth quarter, 1981, will continue in 1982. A study to determine the effect of a low temperature filler sweep before pyrolysis and/or sweeping with heated gas on pyrolyzate activity is planned in the third or fourth quarter, 1982, followed by investigations on preheated filler. Additional testing in 1982 may include the pyrolysis of LTF + trp for the quantitation of carbolines (*versus* activity), and an investigation into fractional pyrolysis (collecting fractions as a function of temperature) in conjunction with personnel in the Analytical Division involved with studies of evolved gas analysis.

E. Filler Precursors of CSC Activity (with 6908 and 6910)

Research in this area is an outgrowth of our longstanding interest in trying to evaluate tobacco fractions to see which, if any, may be major nitrogen-containing precursors of IT CSC activity. In 1981, our efforts were concentrated in the following areas: 1) the effects of salts added to filler; 2) model cigarette additives; and 3) the pyrolysis of cigarette filler (discussed previously in Section D). In 1982, we plan to continue: 1) the salt effects studies (KOAc + RL base web) by measuring recovered CSC activity in base fractions, and 2) the model cigarette additive studies with sugars, amino acids, and proteins on LTF filler.

F. HPLC: Testing of Active Fractions (with 6908 and 6902)

In order to further our efforts to isolate and identify the active components in burley CSC base fractions, we plan to initiate a study in the first quarter, 1982, to test the activity of peaks (representing less than one mg of material) generated by the HPLC of active base fractions of interest. It has been reported that a similar method has been set up and is being used in conjunction with the *Salmonella typhimurium* 8-azaguanine forward mutation assay at M.I.T. Currently, it is planned that a member of project 6902 will visit this laboratory at M.I.T. in order to bring back the 8-azaguanine assay and the methodology for HPLC testing. Members of 6908 will take this methodology (modifying it if necessary for applications in this laboratory) and generate the samples for us to test in the standard *Salmonella*/microsome assay.

G. Denitrated RL Studies

Studies on the effect of various processing parameters and additives on the IT CSC activities of autogenous denitrated RL samples were initiated in 1981, upon the request of R. D. Carpenter and will be continued in 1982. This testing will be done on a prompt and high priority basis.

H. Water Expansion and Related Studies (with G. Keritsis)

In previous years, we have tested bright tobacco expanded by all of the current expansion processes. In 1981, we obtained control and water expanded (steam) uncased bright samples whose IT CSCs showed no significant difference in activity when tested in the *Salmonella*/microsome assay. Because we had never tested expanded burley tobacco, we requested control and water expanded cased and uncased burley machine made cigarettes for testing in the assay. Since the samples that we received were all diluted (between 10 to 15 percent), the samples were tested with and without dilution so that our interpretation of the results was not further complicated by this additional variable. The results showed: 1) the activity of uncased burley was significantly more active than cased burley, with or without dilution; 2) the activity of the diluted samples was significantly different--uncased control>uncased expanded>cased control>cased expanded; and, 3) the activity of the undiluted samples only showed the casing effect and did not show any significant difference in activity between the uncased (control and expanded) samples (like the bright studies), or between the cased (control and expanded) samples. These results were significant because it was the first time that we had seen a casing effect on CSC activity, and we are planning to continue studies in this area in 1982. These results also pointed to the fact that we need to look at some of the cigarette parameters which could explain these results based on differences in smoking characteristics such as coal temperature.

We plan to write a research proposal in the second quarter, 1982, offering an experimental design for evaluation of the effect of a number of filler and construction parameters on CSC activity, (*e.g.*, effect of dilution, coal temperature, rod weight, RTD, firmness, cuts per inch, *etc.*, on IT CSC activity), so that we can request that the appropriate cigarettes be made, and we can begin testing by the fourth quarter, 1982.

I. CSC + Mutagen Study (with 6902 and 6904)

To begin with, a joint research proposal aimed at a broad approach to this problem will be written by the end of the first quarter, 1982. Once this proposal has been worked out and the mutagens/CSCs to be used are selected, we plan to initiate this study in the *Salmonella*/microsome assay by testing mutagens added to CSC (first quarter, 1982). This study will then continue and will be further expanded to CSCs from filler that has been treated with a known mutagen prior to smoking. Our efforts in this area are dictated by the high priority of this study and will be blocked off into a logical sequence of steps so that testing can be achieved as add-ons to our routine testing schedule.

J. CSC Fractions and Model Compounds (with 6908)

Work on isolating and identifying the active components in burley CSC basic fractions (LH-20 fractions from toluene/aqueous methanol extracts) will continue in 1982, along with the model compound studies (Glu-P-1/Glu-P-2; IQ). Beginning in the second or third quarter, we will test fractions from a new preparative gel permeation chromatography procedure to determine how the activity separates and what the differences, if any, there are in comparison to the LH-20 procedure. Testing of acid/neutral fractions to look for any interactive effects (isolate/identify possible active compounds and/or inhibitors) should proceed in the second quarter, 1982. Studies to look at some biochemical interactions, *e. g.*, microsome independent activity of IT *versus* ECT fractions from high nitrate cigarettes in tester strain TA100, are planned in the second or third quarter, 1982. In addition, the testing of PAH fractions from model cigarettes should be completed by the end of the first quarter, 1982.

K. Effect(s) of Nicotine on Activity (with 6908)

We plan to initiate this study in the first quarter, 1982. The study as currently outlined will proceed in three defined phases: 1) to determine the direct effect of nicotine on bright and burley base fraction activity; 2) to determine the direct effect of nicotine on CSC and acid/neutral fraction activity; and, 3) to determine the effect of nicotine in tobacco on the activity of CSC fractions. We anticipate that this high priority study will be completed in the third quarter, 1982. Beginning sometime in the second or third quarter, 1982, we will also begin testing LTF + nicotine samples in conjunction with the proposed aza-arene study (6908).

3. SUMMARY--PLANS AND GOALS FOR 1982

Salmonella/Microsome Mutation Assay	Time
A. Assay Standardization and Quality Assurance	
-Preparation of new TA98 frozen ampoule culture stock for routine inoculum	First quarter
-Obtain/evaluate new batch of Aroclor®-induced rat liver microsomes for routine testing	First quarter
-Evaluate hamster liver microsomes	First quarter
-Make the transition from the Sigma to the DEC system	Fourth quarter, 1981
-Finish the interface of the automatic colony counter to the main computer	Second - Third quarter
-Issue Methods Manual	Second quarter
-Monitor assay response	Continuing
-Test new strains	When available
B. Additive and CSC Testing	Upon request by 6900 project personnel
C. Sidestream *versus* Mainstream Studies	
-Standardize method of sidestream TPM collection, extraction, and preparation of stock solutions in DMSO	First quarter
-Retest 2R1, RCB, burley, bright, and RL reference cigarettes	Second - Third quarter
-Test other cigarette types	Third - Fourth quarter
-Test sidestream fractions: acid/neutral, base, 2AC/H/NH	Second - Third quarter
D. Cigarette Filler Pyrolysis	
-Pyrolyzate *versus* atmosphere and temperature for model cigarette types and filler types	First quarter
-Base fraction: Pyrolyzate *versus* IT CSC activity; also, acid/neutral studies	Second quarter and continuing
-Effect of low temperature sweep and/or sweeping with heated gas on pyrolyzate activity	Third - Fourth quarter

D.	Cigarette Filler Pyrolysis *(continued)*	
	-Fractional pyrolysis	Fourth quarter
	-Pyrolysis of salt effect samples	First quarter
	-Pyrolysis of bright, burley, and 1:1 blend	Fourth quarter, 1981 and continuing
	-Investigations of preheated filler	Third - Fourth quarter
	-Dry filler *versus* conditioned filler	First quarter
E.	Filler Precursors of CSC Activity	
	-Salt effects (KOAc + RL base web): base fractions	Continuing
	-LTF studies: LTF + sugars, amino acids, and/or proteins	Third - Fourth quarter
F.	HPLC: Testing of Active Fractions	First quarter and continuing
G.	Denitrated RL IT CSC Studies: testing of autogenous denitration samples; additives; and processing parameters	Continuing--by request (6900)
H.	Water Expansion Studies	
	-Cased *versus* uncased burley	Continuing
	-Effect of filler and construction parameters on coal temperature and CSC activity	
	--Research Proposal	Second quarter
	--Sample Evaluation	Fourth quarter
I.	CSC + Mutagen Study	
	-Research Proposal	First quarter
	-Mutagen added to CSC	First quarter
	-Mutagen added to filler → test CSC	Second quarter and continuing

J.	CSC Fractions and Model Compounds	
	-Burley CSC base fraction	
	--LH-20 fractions from toluene/aq. MeOH extracts	Continuing
	--Preparative GPC and/or Chromatotron procedure	Second - Third quarter
	--Model compounds: Glu-P-1/Glu-P-2; IQ	Second - Third quarter
	-Test acid/neutral fractions for interactive effects: isolate/identify possible active compounds and/or inhibitors	Second quarter and continuing
	-Biochemical Interactions: microsome independent activity of IT *versus* ECT fractions from high nitrate cigarettes (TA100)	Second - Third quarter
	-PAH fractions from model cigarettes	First quarter
K.	Effect(s) of Nicotine on Activity	
	-Direct effect on base fraction activity	First quarter
	-Direct effect on CSC and acid/neutral fraction activity	First quarter
	-Effect of nicotine in tobacco on the activity of fractions	Second - Third quarter
	-Nicotine aza-arene study (LTF + nicotine)	Second - Third quarter and continuing

Karen Rapp Sherwood

nwp